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Method to Preselect the Sex of Offspring

_Su,Cl_ v/c   **Background of the Invention**

Field of the Invention

This invention relates to a method of preselecting the sex of offspring by sorting sperm into X and Y chromosome-bearing sperm based on differences in DNA content.

Description of the Prior Art

Gender of animal offspring is important to livestock producers. Because the dairy farmer has little use for most bull calves, the use of sexed semen to produce only females would make milk production more efficient. Swine farmers would produce pork more efficiently if they were able to market only female swine, because females grow faster than males.

In beef cattle and sheep breeds, the male grows at a faster rate than the female and hence is preferred for meat production.

In addition, the ability to specify male or female offspring should shorten the time required for genetic improvements, since desirable traits are often associated with one or the other parent. Planning the sex of cattle offspring is already practiced on a limited basis. This procedure consists of removing embryos from the cow, identifying their potential gender, and re-implanting only those of the desired gender. However, an ability to separate sperm into male-producing and female-producing groups before they are used for artificial insemination could enhance the overall value of offspring produced by embryo transfer.

Every living being has a set of paired chromosomes, which carry all the genetic material necessary to maintain life and also to propagate new life.

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All but one pair of chromosomes are called autosomes and carry
genes for all the characteristics of the body, such as skin, hair and
eye color, mature size, and body characteristics. The remaining pair
are called sex chromosomes. They carry the genetic material that
5   specifies gender. One sex chromosome is called X, the other Y.

A sperm from the male or an egg from the female contains one of
each pair of autosomes; in addition, in mammals the egg always
contains an X chromosome, while the sperm always carries either an X
or Y chromosome.

10   When a sperm and egg unite and the sperm carries the Y
chromosome, the offspring is male (XY); however, if the sperm carries
an X chromosome when it unites with the egg, the resulting offspring
is female (XX).

The only established and measurable difference between X and Y
15   sperm that is known and has been proved to be scientifically valid is
their difference in deoxyribonucleic acid (DNA) content. The X
chromosome is larger and contains slightly more DNA than does the Y
chromosome. The difference in total DNA between X-bearing sperm and
Y-bearing sperm is 3.4% in boar, 3.8% in bull, and 4.2% in ram sperm.

20   The amount of DNA in a sperm cell, as in most normal body cells,
is stable. Therefore, the DNA content of individual sperm can be
monitored and used to differentiate X- and Y-bearing sperm.

Since the difference in DNA mass in the sex chromosomes of most
mammals is the only scientifically validated, measurable difference
25   between X- and Y-bearing sperm, the chromosomal constitution
[Moruzzi, J. Reprod. Fertil. *57*: 319 (1979)] and/or measurement
of DNA mass [Pinkel et al. (1), Science *218*: 904 (1982); Pinkel
et al. (2), Cytometry *3*: 1 (1982); Johnson and Pinkel, Cytometry
7: 268 (1986); Johnson et al. (1), Gam. Res. *16*: 1 (1987);
30   Johnson et al. (2), Gam. Res. *17*: 203 (1987)] are the only
verifiable means other than fertility for determining the
sex-producing capability of a population of sperm. The literature

describes many physical, biochemical, and functional methods that
have purportedly sexed sperm [Amann and Seidel, "Prospects for Sexing
Mammalian Sperm," Colorado Assoc. Univ. Press, Boulder (1982)];
several of these methods have been tested for relative DNA content
5 [Pinkel et al., J. Anim. Sci. *60*: 1303 (1985); Johnson (1),
Theriogenology *29*: 265 (1988)].   However, no method has been
proven in controlled experiments to actually affect the sex ratio of
offspring.

Previous studies have demonstrated that the difference in DNA
10 content between X and Y chromosome-bearing sperm can be repeatedly
measured and the sperm sex ratio of a sample of semen predicted
[Johnson and Pinkel, *supra*; Johnson et al. (1), *supra*;
Johnson et al. (2), *supra*; Johnson (1), *supra*; Johnson (2),
Cytometry, Suppl. *2*: 66 (Abstract) (1988)]. Verifiable
15 separation by sorting of X and Y sperm based on DNA content has been
accomplished with the vole [Pinkel et al. (1), *supra*; Johnson,
In "Beltsville Symposia in Agricultural Research X," P. C.
Augustine, H. D. Danforth, & M. R. Bakst (eds.), Martinus Nijhoff,
Boston, pp. 121-134 (1986)] and the chinchilla (Johnson et al. (1),
20 *supra*]. However, preparation procedures damaged DNA viability.
The sorting of sperm nuclei from several mammalian (bull, boar, ram,
vole, chinchilla) species into separate X and Y chromosome-bearing
populations at purities ranging from 92 to 99% has been accomplished
[Johnson and Clarke, Gam. Res. *21*: 335 (1988)]. Nuclear
25 decondensation and pronuclear development was demonstrated in hamster
eggs that had been microinjected with sorted X- or Y-bearing bull,
boar, or ram sperm [Johnson and Clarke, *supra*].

Summary of the Invention

It is an object of this invention to provide a method for sorting
30 mammalian sperm into X and Y chromosome fractions based on DNA
content.

It is a further object of this invention to teach a method of staining the DNA of mammalian sperm while maintaining viability of the sperm.

It is a further object of this invention to provide a sheath fluid adapted to be used in a cell-sorting apparatus while maintaining viability of sperm cells.

It is a further object of this invention to provide a collecting fluid capable of maintaining the viability of sorted sperm cells.

Other objects and advantages of this invention will become readily apparent from the ensuing description.

DE, Cl v/c   **Detailed Description of the Invention**

I have now demonstrated the separation, by flow sorting, of intact, viable X and Y chromosome-bearing rabbit and swine sperm populations based on relative DNA content; surgical insemination of the sorted sperm into does; and the subsequent birth of sexed offspring with a phenotypic sex ratio consistent with predictions based on the relative DNA content of the sorted sperm populations.

A flow cytometer measures the amount of fluorescent light given off when the sperm, previously treated with a fluorescent dye, pass through a laser beam. The dye binds to the DNA. The fluorescent light is collected by an optical lens assembly; the signal is transported to a photomultiple tube, amplied, and analyzed by computer. Because the X chromosome contains more DNA than the Y chromosome, the female sperm (X) takes up more dye and gives off more fluorescent light than the male sperm (Y).

For small differences in DNA to be detected between X and Y, the sperm must pass single file through the laser beam, which measures the DNA content of individual sperm.

In orthogonal flow cytometry, a suspension of single cells stained with a fluorochrome is made to flow in a narrow stream intersecting an excitation source (laser beam). As single cells pass through the beam, optical detectors collect the emitted light, convert the light to electrical signals, and the electrical signals

are analyzed by a multichannel analyzer. The data are displayed as multi- or single-parameter histograms, using number of cells and fluorescence per cell as the coordinates.

In order to use an orthogonal flow cytrometric system to differentiate between X- and Y-bearing sperm DNA, a beveled sample injection tip and a second fluorescence detector in the forward position is required [Johnson and Pinkel, *supra*]. This paper is herein incorporated by reference. The modified system allows one to control the orientation of the flat ovoid sperm head as it passes the laser beam. Elimination of the unoriented sperm by electronic gating enhances precision. Typically, 80% of sperm nuclei (without tails) are properly oriented as they pass the laser beam.

In the modified Epics V flow cytometer/cell sorter, hydrodynamic forces exerted on the flat, ovoid mammalian sperm nuclei orient the nuclei in the plane of the sample stream as they exit the beveled injection tip. Fluorescent signals are collected simultaneously by 90 and 0 degree optical detectors, from the edge and flat side of the sperm nucleus, respectively. For sorting, the sample stream is broken into uniform droplets by an ultrasonic transducer. Droplets containing single sperm of the appropriate fluorescence intensity are given a charge and electrostatically deflected into collection vessels. The collected sperm nuclei then can be used for microinjection into eggs. Since the sperm nuclei have no tails, they cannot be used for normal insemination.

Accurate measurement of mammalian sperm DNA content using flow cytometry and cell sorting is difficult because the sperm nucleus is highly condensed and flat in shape, which makes stoichiometric staining difficult and causes stained nuclei to have a high index of refraction. These factors contribute to emission of fluorescence preferentially from the edge or thin plane of the sperm nucleus. In most flow cytometers and sorters, the direction of sample flow is

orthogonal to the direction of propagation of the laser beam and the optical axes of the fluorescence detection. Consequently, fluorescence measurement is most accurate when the sperm fluorescence is excited and measured on an axis perpendicular to the plane of the sperm head [Pinkel et al. (2), *supra*]. At relatively low sample flow rates, hydrodynamics are used to orient tailless sperm so that DNA content can be measured precisly on 60 to 80% of the sperm passing in front of the laser beam. The modified Epics V system used in this study can measure the DNA content of tailless sperm from most species at the rate of 50 to 150 sperm per second [Johnson and Pinkel, *supra*].

Intact sperm (with tails), whether viable or nonviable, cannot be oriented as effectively as tailless sperm nuclei [Johnson (2), *supra*]. However, a 90-degree detector can be used to select the population of properly oriented intact sperm to be measured by the 0 degree detector. Since no hydrodynamic orientation is attempted, the sample flow rate can be much higher, which compensates somewhat for the fact that only 15 to 20% of intact sperm pass through the laser beam with proper orientation. In this invention, the overall flow rate was approximately 2500 intact sperm per second. The intact X and Y-bearing sperm fractions were sorted simultaneously from the population of input sperm at a rate of 80-90 sperm of each type per second.

It is, of course, of critical importance to maintain high viability of the intact sperm during the sorting process and during storage after sorting but prior to insemination.

Of the factors involved in maintaining sperm viability, the method of staining, the sheath fluid, and the collecting fluid have been found to be especially important.

A nontoxic DNA stain must be selected. A preferred stain is Hoechst bisbenzimide H 33342 fluorochrome (Calbiochem-Behring Co., La Jolla, CA). To our knowledge, this fluorochrome is the only DNA

binding dye that is nontoxic to sperm. Concentration of the
fluorochrome must be minimal to avoid toxicity, and yet be sufficient
to stain sperm uniformly and to detect the small differences in the
DNA of X and Y sperm with minimal variation. A suitable
concentration was found to be 5 μg/ml, but this may be varied
from 4 to 5 μg/ml.

The sperm must be incubated with stain at sufficient temperature
and time for staining to take place, but under mild enough conditions
to preserve viability. Incubation for 1 hr at 35° C was found to
be acceptable, but ranges of 30 to 39° C would also be
effective. Incubation time has to be adjusted according to
temperature; that is, 1.5 hr for 30° C; 1 hr for 39° C.

Sheath fluid used in sorting cells must be electrically
conductive and isotonic. A concentration of 10 mM phosphate buffered
saline provided the necessary electrical properties, and 0.1% bovine
serum albumin was added to enhance sperm viability by providing
protein support for metabolism and viscosity for the sperm. The
sheath fluid must be free of sugars and excess salts.

Dilution of sperm as occurs in sorting tends to reduce viability
of the cells. To overcome this problem, sperm were collected in test
egg yolk extender [Graham et al., J. Dairy Sci. 55: 372 (1972)]
modified by adjusting the pH and adding a surfactant. Details of the
composition of the extender are shown in Example 1. The surfactant
is believed to enhance capacitation of the sperm prior to
fertilization.

To confirm the DNA content and predict the sex of the offspring
of surgically inseminated X or Y sorted sperm fractions, an aliquot
of the sorted sperm was sonicated to remove the tails, stained, and
the nuclei was reanalyzed for DNA content to predict the proportion
of X and Y sperm.

Although the detailed description which follows uses the sorting
of rabbit sperm as an example of this invention, it is expected that

the sperm of most mammals could be effectively sorted by following these procedures. Those skilled in the art will recognize that minor modifications may be made in the procedure without departing from the spirit and scope of the invention.

5   Rabbit semen was collected, diluted, and stained with a fluorochrome dye. Sperm were sorted in a modified Epics V flow cytometer/cell sorter.

After being sorted, sperm were surgically inseminated into the uteri of rabbits.

10.   The results obtained by surgical insemination of does with sorted intact sperm are presented in Table I. Recovery of ova 40 hr post-insemination indicated that stained sorted sperm, as well as unstained unsorted sperm, were capable of fertilizing rabbit ova *in vivo*.

15   Inseminations were also made to determine the comparability of predicted sex of offspring to phenotypic sex. As the data in Table II indicate, the predictability of the phenotypic sex based on DNA analysis of the separated intact sperm was very high. Reanalysis of the sorted Y population used for insemination indicated that 81% of

20   the sperm were Y-bearing. The sex ratio of offspring from these inseminations was identical to that predicted. These values were

21 significantly different from theoretical 50:50 sex rates (P <.003). Reanalysis of the sorted X-bearing sperm population used for insemination indicated that 86% were X-bearing and 14% were Y-bearing

25   sperm. The phenotypic sex of the offspring from these inseminations was 94% female, which was different from the theoretical 50:50

21 (P <.0003).

Inseminations were made with sorted X and Y populations that were recombined (recombined X and Y group) immediately before

30   insemination. The assumption was made that the proportions of X and Y in the recombined samples were equal (50:50). The phenotypic sex resulting from the inseminations was 57% female and 43% male (Table II) and was not significantly different from the theoretical (50:50) sex ratio (P = 0.40).

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Table I. Fertilizing Capacity of Flow-Sorted Rabbit
Spermatozoa After Intrauterine Insemination of Does

| Treatment of Sperm | Number of | | | |
	Does Inseminated	Ovulation Points	Eggs Recovered	Eggs Fertilized
Unsorted	2	16	9	9
Sorted	6*	59	46	39

*One doe accounted for 7 recovered and 7 unfertilized eggs.

Table II. Predicted and Actual Sex Ratios of Offspring After Intrauterine Insemination of Sorted X and Y Chromosome-Bearing Rabbit Sperm

Treatment of Sperm	Number of Does		Total No. of Young Born	Percentage and Numbers of Offspring			
	Inseminated	Kindling		Predicted		Actual	
				% Males	% Females	% Males (N)	% Females (N)
Sorted Y	16	5	21	81	19	81 (17)	19 (4)
Sorted X	14	3	16	14	86	6 (1)	94 (15)
Recombined X and Y	17	5	14	50	50	43 (6)	57 (8)
Total	47	13	51	—	—	47 (24)	53 (27)

sex ratio (P = .40). The phenotypic sex ratio of offspring born of does inseminated with either sorted X-bearing or sorted Y-bearing sperm was different (P < .0002 for X and P < .001 for Y) from the theoretical (50:50) sex ratio expected from untreated semen.

Embryonic mortality was significant in the does inseminated with sorted intact sperm. With a reasonably high fertilization rate (Table I), one would expect a kindling rate of near 80% and litter size of about six from does of this age and breed. However, the kindling rate across the three treatment groups averaged 28%, with an average litter size of 3.9. The cause of the apparent high rate of embryonic death is thought to be due to the fluorochrome binding to the DNA and/or to the effect of the laser beam exciting the DNA bound fluorochrome. Earlier work has shown that sorted vole sperm nuclei that were microinjected into hamster eggs exhibited chromosome breakage in the developing sperm pronucleus [Libbus et al., Mut. Res. *182*: 265 (1987)]. Those sperm had been sonicated, stained, sorted, and microinjected, a somewhat more rigorous treatment than the staining and sorting used in this study.

I have demonstrated that DNA can be used as a differentiating marker between X- and Y-bearing sperm, that DNA can be used to accurately predict the sex of offspring from separated X- and Y-bearing sperm populations, and that flow sorting is an effective means for separating viable X- and Y-bearing sperm populations suitable for production of offspring.

The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention, which is defined by the claims.

Example 1

Semen was collected from mixed breed mature bucks by use of an artificial vagina. Sperm concentration was determined with a hemocytometer. The semen was diluted with Tris buffer, pH 6.9, to a concentration of 10×10^6 per ml. Bisbenzimide H 33342

fluorochrome was added at a concentration of 5 μg/ml. The samples were incubated for 1 hr at 35° C. Intact sperm were sorted on a modified EPICS V flow cytometer/cell sorter. The stained intact sperm were excited in the ultraviolet (UV; 361 and 364 nm) lines of a 5-watt 90-5 Innova Argon-ion laser operating at 200 mW. Data were collected as 256-channel histograms. Sheath fluid was 10 mM phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Sperm were sorted into a test egg yolk extender.

The composition of the extender was N-tris(hydroxymethyl) methyl-2-amino ethane sulfonic acid, 2.16 g; tris hydroxymethyl aminomethane, 0.51 g; dextrose, 0.1 g; streptomycin sulfate, 0.13 g; penicillin G, 0.08 g; egg yolk, 12.5 ml; Equex STM (Nova Chemical Sales, Scituato, MA), 0.5%; and distilled water, 50 ml. This mixture was centrifuged, and only the supernatant was used. The sorted sperm were concentrated by incubating at room temperature for 1 hr, after which the more dilute fraction was removed and the remainder was used for insemination 1 to 4 hr later.

Example 2

Mature New Zealand White does were injected with 150 international units of human chorionic gonadotropin (HCG) to induce ovulation, which was expected to occur 10 hr later. Seven hours after treatment with HCG, the does were surgically prepared by injection with Ketamine hydrochloride containing acepromazine and anesthetized under halothane and oxygen. The uterus was exposed by midline incision, and 100 μl of sorted or unsorted sperm was placed into the lumen of the anterior tip of each uterine horn through a 21-gauge needle. Standard management practices were used in caring for the rabbits. These does were sacrificed 40 hr post-insemination; uteri were flushed and recovered eggs evaluated. All fertilized eggs recovered were classified as morula. The results of these experiments are shown in Table I.

Cℓ ᵛ/c **Example 3**

Table II shows the results of inseminations made into the tip of the uterine horn: the number of does that kindled and the phenotypic sex of the offspring compared to the predicted sex. Predicted sex of

5   offspring was based on reanalysis of sorted intact sperm to determine relative DNA content. For reanalysis, the sorted sperm was sonicated for 10 sec and centrifuged at 15,000 g, the supernatant was discarded, and the pellet was resuspended in 9 μM bisbenzimide H 33342. Phenotypic sex of the offspring was determined soon after

10   birth and confirmed at later ages up to 10 weeks. Recombined X and Y is the sorted X and Y sperm populations recombined immediately before insemination.

Cℓ ᵛ/c **Example 4**

Using the methods of Examples 1, 2, and 3, viable swine sperm was

15   sorted into viable X and Y chromosome-bearing populations. Two litters (18 pigs) from surgically inseminated boar semen produced 88% females from X-sorted sperm and 67% males from Y-sorted sperm.

It is understood that the foregoing detailed description is given mainly by way of illustration and that modification and variation may

20   be made therein without departure from the spirit and scope of the invention.